DOI:
10.1016/j.neuro.2016.09.020

Document Version
Peer reviewed version

Reversal of cardiac vagal effects of physostigmine by adjunctive muscarinic blockade

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Words: 5349 including abstract, references and figure legends

Abstract (242 words)

Pre-treatment with reversible acetylcholinesterase (AChE) inhibitors is an effective strategy for reducing lethality following organophosphate nerve agent exposure. AChE inhibition may have unwanted cardiac side effects, which could be negated by adjunctive anti-cholinergic therapy. The aims of the present study were to examine the concentration-dependent effects of physostigmine on cardiac responses to vagus nerve stimulation (VNS), to test whether adjunctive treatment with hyoscine can reverse these effects and to assess the functional interaction and electrophysiological consequences of a combined pre-treatment. Studies were performed in an isolated innervated rabbit heart preparation. The reduction in heart rate with VNS was augmented by physostigmine (1-1000nmol/L), in a concentration-dependent manner - with an EC_{50} of 19nmol/L. Hyoscine was shown to be effective at blocking the cardiac responses to VNS with an IC_{50} of 11nmol/L. With concomitant perfusion of physostigmine, the concentration-response curve for hyoscine was shifted downward and to the right, increasing the concentration of hyoscine required to normalise (to control values) the effects of physostigmine on heart rate. At the lowest concentration of hyoscine examined (1nmol/L) a modest potentiation of heart rate response to VNS (+15±3%) was observed. We found no evidence of cardiac dysfunction or severe electrophysiological abnormalities with either physostigmine or hyoscine alone, or as a combined drug-therapy. The main finding of this study is that hyoscine, at concentrations greater than 10^{-8}M, is effective at reversing the functional effects of physostigmine on the heart. However, low-concentrations of hyoscine may augment cardiac parasympathetic control.

Keywords
acetylcholinesterase; organophosphate nerve agents; vagus nerve; physostigmine; hyoscine; scopolamine; eserine

1. Introduction

Exposure to highly toxic organophosphate nerve agents results in irreversible inhibition of peripheral and central acetylcholinesterase (AChE) resulting in acetylcholine accumulation and over-stimulation of muscarinic and nicotinic receptors. Symptoms of nerve agent poisoning include hyper-secretion, convulsions, respiratory distress, coma and death.

Numerous studies in non-primates and non-human primates have shown that pre-treatment with reversible cholinesterase inhibitors, with and without anti-cholinergics, improves survival and reduces incapacitation after nerve agent exposure.(Bonhage et al., 2009; Lim et al., 1991; Lim et al., 1988; Muggleton et al., 2003; Philippens et al., 1998; Philippens et al., 2000; von Bredow et al., 1991; Wetherell et al., 2002; Wetherell, 1994) Pyridostigmine (a quaternary compound restricted primarily to the peripheral nervous system) and physostigmine (a neutral compound which can more readily pass the blood brain barrier) are long acting, but reversible, AChE inhibitors that are effective pre-treatments for nerve-agent exposure. While both agents are effective in preventing lethality in animal studies, physostigmine appears to be more effective against incapacitation, most likely as a consequence of its combined central and peripheral actions.(Wetherell et al., 2002)

Common side effects of physostigmine treatment include gastrointestinal irritation, urinary urgency, diaphoresis, headaches and vomiting. Potential cardiac effects are also important and have been examined in a limited number of animal studies, which demonstrate bradycardia and atrio-ventricular block due to the potentiation of parasympathetic nervous control (mediated through the vagus nerve).(James et al., 1979; Lazartigues et al., 1999) Despite this risk, electrocardiogram monitoring has not been routinely performed in pre-clinical studies investigating nerve agent pre-treatment. Co-administration with competitive muscarinic receptor antagonists (e.g. hyoscine) may also reduce symptoms associated with physostigmine treatment and overdose.(Moore et al., 2014)

The aims of the present study were - 1) to examine the concentration-dependent effects of physostigmine on cardiac responses to vagus nerve stimulation (VNS), 2) to test whether adjunctive treatment with hyoscine can reverse the cardiac effects of physostigmine and 3) to assess the functional and electrophysiological consequences of combined drug therapy. Studies were conducted in isolated perfused rabbit hearts with intact autonomic innervation.

2. Methods

2.1 Animal welfare

All procedures were undertaken in accordance with ethical guidelines set out by the UK Animals (Scientific Procedures) Act 1986 and Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Studies conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health under assurance number A5634-01. Experiments were performed in white adult New Zealand rabbits (3.0-4.5kg, n=24)

2.2 Innervated isolated heart

The studies in this report used the isolated innervated rabbit heart as described by Ng *et al.,* with minor modifications.(Ng et al., 2001) Animals were pre-sedated using a mixture of ketamine (Ketaset, 10mg/kg, Fort Dodge, UK), medetomidine hydrochloride (Sedator, 0.2mg/kg, Dechra, UK) and butorphanol (Torbugesic, 0.05mg/kg, Fort Dodge, UK) (i.m.). Anaesthesia was induced and maintained using propofol (5mg as required, Rapinovet, Schering-Plough Animal Health, UK) with concomitant heparin (1000 units, Multiparin, UK). Animals were intubated and ventilated at 60 breaths per minute of room air. The major blood vessels leading to and from the thorax were ligated and cut. Animals were then killed by an overdose of sodium pentobarbitone solution (160mg/kg i.v.), the descending aorta was cannulated and the preparation rapidly excised from C1-T12 (on ice). The preparation was moved to the perfusion apparatus and retrogradely perfused under conditions of constant pressure with an oxygenated ($95\%0_2/5\%CO_2$) Krebs-Henseleit buffer solution containing (in mM): Na^+ 140.0, K^+ 4.0, Ca^{2+} 1.8, Mg^{2+} 1.0, HCO_3^- 24.0, $H_2PO_4^-$ 0.4, Cl^- 121.0, glucose 11.0 and pyruvate 2. Aortic perfusion pressure was recorded using a pressure transducer placed in series with the perfusion line and was maintained at 80±5mmHg using a feedback system (STH Pump Controller, ADInstruments, Oxford, UK) controlling a peristaltic pump (D23-Du, Watson-Marlow, Cornwall, UK). Left ventricular pressure was assessed using fluid filled balloon inserted to the left ventricular cavity. Balloon volume was increased to give a left ventricular end diastolic pressure (LVEDP) of 5mmHg. Balloon volume was not altered for the remainder of the experiment and hearts were allowed to beat at their intrinsic sinus rate. Bipolar electrograms were recorded with one electrode attached to the LV apex and one electrode placed on the thorax. Signals were filtered using a band-pass filter of 0.1-200Hz (1kHz acquisition).

2.3 Vagus nerve stimulation

The right vagus nerve was dissected from the surrounding tissues and placed upon custom-made silver wire electrodes, connected to a constant voltage optically isolated stimulator. Nerves were stimulated at 3X threshold voltage

with 2ms square wave pulses. Threshold voltage was defined as the minimum voltage required to lower heart rate by 10bpm from baseline values. The frequency of VNS was chosen as that which gave a stable reduction in heart rate of approximately 80 to 100bpm. A greater magnitude response was accepted if stability was deemed to be poor at lower intensities of stimulation. In the event that the right vagus was unresponsive the left vagus was used. To prevent unwanted activation of the sympathetic nervous system the spinal cord was removed in all experiments.

2.4 Solutions

Physostigmine (physostigmine salicylate salt, 45720, Sigma-Aldrich, UK) and hyoscine (hyoscine hydrobromide, S0929, Sigma-Aldrich, UK) were added, from concentrated 1mmol/L stock solutions, to a 5-liter volume of Krebs-Henseliet buffer just prior to perfusion. Stock solutions were freshly prepared each day.

2.5 Study protocols

1. In study protocol 1 the effects of increasing concentrations of physostigmine and hyoscine on cardiac functional and electrophysiological parameters and responses to VNS were investigated in separate hearts (n=8 in each group). Following a 30-minute stabilization period, hearts were perfused with incremental concentrations of physostigmine or hyoscine (1-1000nmol/L). Responses to VNS (2-minute stimulation) were tested 10-minutes after initiating drug perfusion.
2. Study protocol 2 examined the effect of increasing concentrations of hyoscine (1-300nmol/L) on functional and electrophysiological parameters in the presence of 2, 20 or 200nmol/L physostigmine (as defined by study protocol 1). Studies were designed to examine the facility of hyoscine in reversing the cardiac effects of physostigmine. (n=8 in each group)

2.6 Analysis and statistics

Data were digitized at 1kHz on a 16-channel PowerLab recording system and associated LabChart software (v8, ADInstruments, UK). Baseline data represent the mean of each variable over a stable 20-second period just prior to VNS. Responses to VNS were measured over the 20-second period just prior to the cessation of nerve stimulation. Electrocardiogram parameters represent the mean of 10 consecutive beats. Heart rate corrected QT index was calculated from linear regression of QT interval and heart rate from baseline data (see Supplementary Figure 1) using the formula QT measured / QT expected *100. Data for predicted QT calculations were taken from values following the 30-minute stabilization period from n=12 hearts used in physostigmine and hyoscine concentration-response experiments. Reversal

concentration was defined as the concentration of hyoscine required to return heart rate responses to VNS in the presence of physostigmine to preceding baseline values. Hill slope, IC_{50} and reversal concentration were calculated for each experiment by fitting data to the following model (GraphPad Prism v6.0).

$$Y = Bottom + (Top-Bottom) / (1+10^{((Log\ IC_{50} - X) * HillSlope)})$$

Where Top and Bottom are plateaus in the units of the Y-axis (unconstrained). Statistical analysis was conducted by paired Student's t-test (repeated measures in the same heart) and 1- or 2-way repeated measures ANOVA with Bonferonni post hoc tests, as appropriate (GraphPad Prism v6.0). Significance was set at $P<0.05$. Presented data represent mean±SEM.

3. Results

3.1 Physostigmine alone

The effect of increasing concentrations of physostigmine on cardiac responses to VNS are presented in Figures 1 and 2. During VNS, a concentration-dependent slowing of heart rate was observed with increasing levels of physostigmine (see example traces and mean data in Figure 1A, 1B and 1C). The maximal effect equated to a 28±5% greater reduction in heart rate in the presence of physostigmine (Figure 1B). At concentrations of 100nmol/L and greater, physostigmine also caused a slowing of the intrinsic (baseline) heart rate (Figure 1D). As a result, the absolute change in heart rate (i.e. baseline minus VNS) demonstrated a bi-phasic relationship (Figure 1E), with an initial increase, attributed to the greater slowing of heart rate during VNS, followed by a decrease, with higher concentrations of physostigmine, when the intrinsic heart rate began to fall. Relative heart rate during VNS, expressed as a percentage of control values, demonstrated a sigmoidal relationship - with an IC_{50} of 19nmol/L. At the top end of the physostigmine concentration-range we observed a slowing on the rate of recovery with the cessation of nerve stimulation. This is evidenced by the increase in time to 75% recovery of heart rate following VNS in Figure 1F (see also example traces in Figure1A). Fasciculations of the skeletal muscles of the neck and thorax were commonly observed with physostigmine concentrations in the range of 500-1000nmol/L.

Peak left ventricular pressure (LVP) was stable throughout testing (Figure 1G), with a negligible increase in left ventricular end diastolic pressure (LVEDP) over the study protocol (data not shown). VNS was associated with an increase in peak LVP, the magnitude of which decreased over the course of the experiment with increasing concentrations of physostigmine (Figure 1G, overall effect; $P<0.01$).

VNS was associated with an increase in the QT interval (Figure 2A, overall effect; $P<0.0001$), with no change in QT index (Figure 2B), indicating that QT prolongation during VNS occurred as a consequence of the slowing of heart rate. The QT interval prolonged with increasing concentrations of physostigmine, in a manner related to the slowing of the intrinsic (baseline) heart rate and, with high-concentrations of physostigmine, rate-independent QT prolongation (see the modest increase in the heart rate corrected QT index with 1000nmol/L physostigmine in Figure 2B). There was no effect of physostigmine on PR interval at baseline or with VNS (Figure 2C).

3.2 Hyoscine alone

Data on the effect of increasing concentrations of hyoscine on cardiac responses to VNS are presented in Figures 3 and 4. During VNS, heart rate was lower with low-dose hyoscine perfusion (1nmol/L) in 6 of 8 hearts studied,

however, there was no overall difference in the magnitude of heart rate slowing between conditions (changes from baseline; 87±5 vs. 75±4bpm, P>0.05). With higher concentrations of hyoscine, the magnitude of slowing in heart rate with VNS was reduced, and heart rate was elevated compared to VNS in control conditions (see example traces and mean data in Figure 3A, 3B and 3C). Baseline heart rate was unaffected by hyoscine perfusion (Figure 3D). Both the absolute heart rate observed during VNS (expressed as % of control values), and the change in heart rate from baseline values (baseline minus VNS), demonstrated a sigmoidal concentration-response, with IC_{50} values of 11 nmol/L (Figure 3C&E).

The positive inotropic response (Figure 3F) and prolongation of QT interval (Figure 4A) with VNS, being a function of heart rate, were abolished with increasing concentrations of hyoscine. LVEDP demonstrated a small increase over the duration of the experiment, similar to that seen with physostigmine (data not shown). Hyoscine had no effect on the heart rate corrected QT index (Figure 4B) or PR interval (4C).

3.3 Combined physostigmine and hyoscine

Data on the effect of increasing concentrations of hyoscine on cardiac responses to VNS, in the presence of physostigmine, are presented in Figures 5-7. High concentration physostigmine (200nmol/L) was associated with a slowing of intrinsic (baseline) rate and heart rate during VNS (see Figure 5). A similar trend for slower heart rates during VNS was observed with a medium (20nmol/L) concentration of physostigmine (P=0.059). Low-concentration physostigmine (2nmol/L) was not associated with any effect on heart rate. Changes in peak LVP mirrored those seen in heart rate, whereby a reduction in rate due to VNS was associated with an increase in contractile force (Figure 5). As the heart rate response to VNS was inhibited, by increasing concentrations of hyoscine, so was the associated positive inotropic response. [Note: Data from 1 heart were excluded from functional analysis due to a leak in the ventricular balloon].

Normalised concentration-response curves for the effects of hyoscine on heart rate during VNS, with 3 physostigmine concentrations and for controls (from study protocol 1), are presented in Figure 6A. Increasing the background concentration of physostigmine was associated with a downward and rightward shift in the concentration-response curve for hyoscine (expressed as a % of heart rate during VNS in control conditions). As a consequence, the IC_{50} and reversal concentration for hyoscine (i.e. the concentration of hyoscine required to normalise to control values the effects of physostigmine on heart rate) were greater in the presence of physostigmine (Figure 6B&C). In the case of a 20nmol/L physostigmine, a concentration of 12±1nmol/L hyoscine was required

to reverse its effects on heart rate. Hill slopes were comparable between groups (Figure 6D).

In all experimental groups (control, 2, 20 & 200nmol physostigmine), low concentrations of hyoscine (1nmol/L) were found to potentiate the heart rate response to VNS. Of 32 experiments, 28 demonstrated a greater slowing of heart rate following low-concentration hyoscine perfusion (see Figure 7). This equates to a 15±3% greater slowing of heart rate versus VNS in control conditions (paired t-test, $P<0.0001$).

There was no observable effect of combined physostigmine and hyoscine on electrocardiogram parameters, except for the influence of hyoscine on heart rate (data not shown).

4. Discussion

The main finding of this study is that hyoscine is effective at reversing the functional effects of physostigmine on the heart and that combined drug therapy has no obvious adverse effect on left ventricular performance or electrophysiological parameters. However, there are some potential exceptions to this overall conclusion; namely, that low-concentration hyoscine may act to augment cardiac parasympathetic control and the competitive nature of muscarinic antagonism by hyoscine.

The parasympathetic nervous system innervates the heart at the sinoatrial node, atrio-ventricular node and ventricular tissue through the vagus nerve. Acetylcholine, released from efferent nerve endings, binds to muscarinic receptors resulting in pacemaker inhibition and membrane hyperpolarization. This mediates the major actions of the vagus nerve i.e. slowing of heart rate and increased conduction delay through the atrioventricular node (note; the PR interval is affected by both heart rate and the vagus nerve, which act in opposition, and does not change during VNS in the absence of cardiac electrical pacing). Through the baroreceptor reflex the vagus is an important determinant of central blood pressure by feedback regulation of cardiac output. Consequently, effects on cardiac function are one of the most clinically important potential side effects of AChE inhibition. In the present study, we demonstrate a concentration-dependent action of physostigmine on the heart rate responses to VNS in the isolated rabbit heart. As predicted by its cholinergic action, physostigmine potentiated the magnitude of heart rate slowing in response to a fixed intensity of VNS. This is likely due to the prevention of acetylcholine hydrolysis and a resultant increase in the concentration and duration of action of acetylcholine at the synapse. The observation that high-concentration physostigmine lowers intrinsic heart rate implies a basal level of acetylcholine release and degradation in the Langendorff preparation in the absence of nerve stimulation.

With increasing concentrations of physostigmine, a downward and rightward shift in the concentration-response to hyoscine was observed. As a result, a greater concentration of hyoscine was required to offset the cardiac effects of increasing concentrations of physostigmine. This likely reflects that accumulated acetylcholine, secondary to AChE inhibition, acts to compete with hyoscine, a competitive muscarinic antagonist, for binding to the muscarinic receptors. Therefore, the concentration of hyoscine required to overcome the effects of physostigmine will vary depending on the concentration of physostigmine in the blood.

In the present study we observed a small, but statistically significant, augmentation of heart rate responses following perfusion of low-concentration

hyoscine, suggesting a possible selective pre-synaptic effect. There is existing evidence that acetylcholine can bind to pre-synaptic muscarinic receptors, resulting in feedback inhibition of additional acetylcholine release.(Brodde and Michel, 1999; Raczkowska et al., 1983; Wellstein and Pitschner, 1988) Experiments by Wellstein and Pitchner (1988) suggest that this cholinomimetic action may be mediated by type 1 muscarinic receptors (M1), being blocked by pirenzepine, an M1-selective muscarinic antagonist, whereas type 2 muscarinic receptors likely mediate post-synaptic inhibition of responses to VNS.(Brodde and Michel, 1999; Wellstein and Pitschner, 1988) As such, it is possible that low-concentration hyoscine may act to augment the effects of physostigmine, when both drugs are used in combination, by blocking this feedback pathway. This concept requires that hyoscine have a higher affinity for the M1 vs. M2 receptor. Unfortunately, we have been unable to find studies on the binding affinity of hyoscine in tissues from the rabbit. However, Zheng *et al.* have reported binding affinities for hyoscine (also known as scopolamine) to human M1 and M2 receptors, expressed in Chinese hamster ovarian cells.(Zheng et al., 2013) The authors reported Ki values of 7.5 and 9.5nmol/L for the M1 and M2 receptor, respectively, indicating a greater affinity for the M1 receptor. However, these data represent only triplicate measurements and no statistical comparison is provided. Augmentation of responses to VNS by low-concentration hyoscine may present an additional challenge in the presence of higher concentrations of physostigmine (i.e. greater than 10nmol/L). Moreover, low-concentration hyoscine may act to potentiate intrinsic cardiac-parasympathetic reflexes (i.e. in the absence of physostigmine). Given that the present study was performed in isolated rabbit hearts, it is difficult to establish if this small effect represents a clinically significant observation (i.e. whether there is a risk for haemodynamic compromise *in vivo*). However, it should be considered that if physostigmine plasma concentration were to rise more rapidly than that of hyoscine, then it is possible that there may be a transient risk of heart rate slowing, even if the ultimate steady-state concentrations for both these agents have no combined effect on heart rate.

Importantly, we observed no evidence of severe cardiac dysfunction or electrophysiological abnormalities with either physostigmine / hyoscine alone or with combined drug-therapy. The modest prolongation of heart rate corrected QT index at 1000nmol/L physostigmine may indicate off target channel effects, however, this is unlikely to have any functional significance with respect nerve agent pre-treatment. At this concentration range, physostigmine was associated with spontaneous fasciculations of the skeletal muscles of the neck and thorax, indicative of over stimulation of the nicotinic receptors. Clearly, this is far in excess of clinically efficacious plasma concentrations of approximately 100pg/mL.(Möller et al., 1999)

The finding that VNS caused an increase in LVP appears at odds with previous reports suggesting that vagal stimulation decreases ventricular force in the rabbit heart.(Brack et al., 2006; Ng et al., 2001; Winter et al., 2015) However, Ng *et al* (2006) reported a bi-phasic force-frequency response, consisting of an initial increase followed by a secondary decrease in ventricular force with increasing heart rate. (Brack et al., 2006) Baseline heart rates were markedly greater in our study than in previous studies (perhaps due to improved perfusion) and so it stands to reason that hearts may be initially operating on the downward slope of the bi-phasic force-frequency curve.(Brack et al., 2006; Ng et al., 2001; Winter et al., 2015) However, we cannot rule out that the positive inotropic response to VNS reflects the low oxygen carrying capacity of the Krebs buffer and resulting improved energetics when heart rate is slowed. The influence of hyoscine and physostigmine on the inotropic response to VNS are likely to be secondary to effects on heart rate (i.e. a loss of heart rate response to VNS in the case of hyoscine and a fall in baseline heart rate in the case of physostigmine), however, this would need to be confirmed in experiments utilizing electrical pacing to control for the effect of heart rate.

4.1 Study limitations

The choice of a cumulative dosing protocol and the duration of drug perfusion used in the present study was based on observations from preliminary experiments; assessing the stability of nerve responses over time and rate of drug washout. On this basis, experiments were limited to a total duration of 120-minutes. However, we did not perform preliminary experiments to examine the rate of onset of action of either physostigmine or hyoscine, which may influence our results (e.g. accuracy of EC_{50}/IC_{50} estimates). If, for example, the rate of inactivation of AChE was sufficiently slow, the effect of physostigmine at low-doses may have been underestimated. Similarly, we cannot discount cumulative effects - resulting from longer-term exposure to physostigmine or hyoscine. In previous work, we reported the time for inactivation and reactivation of actetylcholinesterase in red blood cells treated with 100nmol/L of physostigmine.(Wetherell and French, 1991) The results showed that acetylcholinesterase activity was reduced by 74% within 3-minutes of the addition of physostigmine, with a peak inhibition of 81% by 14-minutes. Therefore, at least with higher concentrations of physostigmine, near maximal inhibition is likely to be achieved within 10-minutes of drug perfusion – as used in the present study. In previous work, the rate of reactivation of acetylcholinesterase (i.e. decarbomoylation) was substantially slower than the rate of inactivation, with a time course of several hours.(Wetherell and French, 1991) A such, a non-cumulative concentration-response, starting at the highest concentration of physostigmine, would not be possible within a suitable timeframe for experiments in isolated hearts

The isolated innervated heart provides a simplified model system for the study of cardiac parasympathetic control. However, our main finding, that hyoscine can reverse the neuro-cardiac actions of physostigmine, does not necessarily predict the action of physostigmine and hyoscine in the intact whole system and should be confirmed by *in vivo* studies - either by direct stimulation of the parasympathetic vagus nerve or by other methods. One alternative approach would be to assess baroreceptor reflex gain before and after drug exposure, by use of vasoactive compounds (e.g. phenylephrine), and the simultaneous measurement of systemic blood pressure and heart rate.(Wang, 1994) Beat-to-beat oscillations in heart rate, known as heart rate variability, can also be used to assess the level of sympathetic and parasympathetic tone, albeit indirectly.(Stein et al., 1994) Alternatively, direct electrical recordings could be made in the sympathetic stellate ganglia and parasympathetic vagus nerve, before and after drug exposure.(Ogawa et al., 2007)

5. Conclusion(s)

In conclusion, we find that hyoscine is a safe and effective agent for the reversal of unwanted cardiac effects of physostigmine treatment. However, there is the possibility, albeit slight, that low-concentration hyoscine may act to potentiate the cardiac actions of physostigimine.

Acknowledgements

All work in this study was funded by the Defence Science and Technology Laboratory, Porton Down, Wiltshire, UK.

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7. Figure legends

Figure 1. Functional responses to vagus nerve stimulation in the presence of increasing concentrations of physostigmine. A) Representative traces demonstrating heart rate response to vagus nerve stimulation (VNS) with 1, 10, 100 and 1000nmol/L physostigmine (in a single experiment). Dotted lines denote 75% recovery of heart rate on the cessation of nerve stimulation. Spikes reflect ectopic beats. B) Mean data demonstrating change in heart rate during VNS with increasing concentrations of physostigmine. C&D) Mean data demonstrating baseline heart rate (C) and heart rate during VNS (D) (expressed as a % of control values). E) Calculated change in heart rate (baseline minus VNS) at each concentration of physostigmine. F) Mean data on time to 75% heart rate recovery following the cessation of VNS with increasing concentration of physotigmine. G) Peak left ventricular pressure (LVP) at baseline and during VNS with increasing concentration of physostigmine. Data represent mean ± SEM. Statistical comparisons by one- (C-F) or two-way (B&G) repeated-measures ANOVA, with Bonferonni post-hoc tests. Different from control (baseline); $^{\wedge}$P<0.05. Different from control (VNS); *P<0.05. Different from 1nmol/L physostigmine; $^{\&}$P<0.05. (n=8).

Figure 2. Electrophysiological responses to vagus nerve stimulation with physostigmine. Mean data of QT interval (A), QT index (B) and PR interval (C) at baseline and just before the cessation of VNS with incremental increases in physostigmine concentration (separate experiments). Data represent mean ± SEM. Statistical comparisons by two-way repeated-measures ANOVA, with Bonferonni post-hoc tests. Different from control (baseline); $^{\wedge}$P<0.05. Different from control (VNS); *P<0.05. (n=8).

Figure 3. Functional responses to vagus nerve stimulation in the presence of increasing concentrations of hyoscine. A) Representative traces demonstrating heart rate response to vagus nerve stimulation (VNS) with 1, 10, 100 and 1000nmol/L hyoscine (in a single experiment). B) Mean data demonstrating change in heart rate with VNS at increasing concentrations of hyoscine. C&D) Mean data demonstrating baseline heart rate (expressed as a % of control values) (C) and heart rate during VNS (D). E) Change in heart rate. F) Peak left ventricular pressure (LVP) at baseline and during VNS with increasing concentration of physostigmine. Data represent mean ± SEM. Statistical comparisons by one- (C-E) or two-way (B&F) repeated-measures ANOVA, with Bonferonni post-hoc tests. Different from control (baseline); $^{\wedge}$P<0.05. Different from control (VNS); *P<0.05. Different from 1 nmol/L hyoscine; $^{\&}$P<0.05. (n=8).

Figure 4. Electrophysiological responses to vagus nerve stimulation with hyoscine. Mean data of QT interval (A), QT index (B) and PR interval (C) at baseline and just before the cessation of VNS with incremental increases in hyoscine concentration (separate experiments). Data represent mean ± SEM. Statistical comparisons by two-way repeated-measures ANOVA, with Bonferonni post-hoc tests. *P<0.05 statistically significant vs. relevant control. (n=8).

Figure 5. Functional responses to vagus nerve stimulation in the presence of physostigmine and increasing concentrations of hyoscine. Mean data demonstrating change in heart rate (left) and peak left ventricular pressure (LVP, right) with vagus nerve stimulation (VNS) in the presence of a fixed concentration of physostigmine and increasing concentrations of hyoscine. Data from 3 groups; A&B) 2nmol/L physostigmine, C&D) 20nmol/L physostigmine and E&F) 200 nmol/L physostigmine. Data represent mean ± SEM. Statistical comparisons by two-way repeated-measures ANOVA, with Bonferonni post-hoc tests. Different from control (baseline): ^P<0.05. Different from control (VNS): *P<0.05. (n=8, all groups)

Figure 6. Concentration-response curves for hyoscine in the presence of physostigmine. A) Mean data demonstrating the effect of increasing concentrations of hyoscine on changes in heart rate during VNS in the presence of 0, 2, 20 and 200nmol/L physostigmine (PHY). Data are normalised as a percentage of the control response in each heart. Control data (0nmol/L) are derived from Study Part A. B-D) Mean data of IC_{50}, reversal concentration and hill slope for hyoscine in each condition. Data represent mean ± SEM. Different from 1nmol/L hyoscine; *P<0.05. Statistical comparisons by one-way repeated-measures ANOVA, with Bonferonni post-hoc tests. Different from control (0nmol/L); ^P<0.05. Different from 2nmol/L physostigmine; $^{\&}$P<0.05. (n=8, all groups)

Figure 7. Potentiation of heart rate responses by low-concentration hyoscine. Scatterplot showing data from 32 experiments, demonstrating a larger magnitude of heart rate slowing with vagus nerve stimulation following perfusion of low concentration (1nmol/L) hyoscine. Data represent the change in heart rate in response to vagus nerve stimulation from control or with 1 of 3 concentrations of physostigmine (2, 20 & 200nmol/L) with and without concomitant low-concentration hyoscine. Statistical comparisons by Student's paired t-tests. Different from control (CNT) / physostigmine (PHY); *P<0.05. Data represent mean ± SEM.

Figure 1

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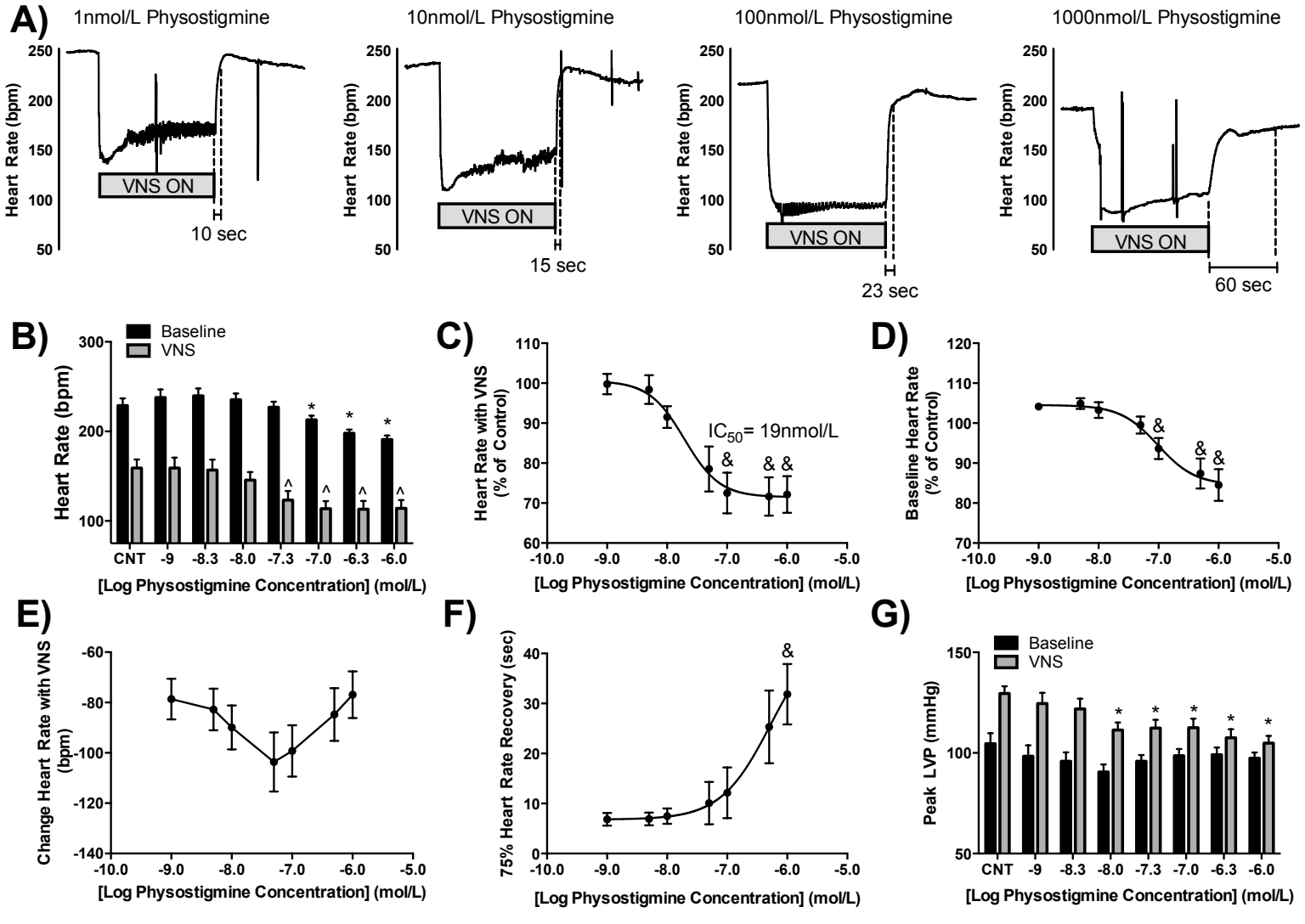

Figure 2

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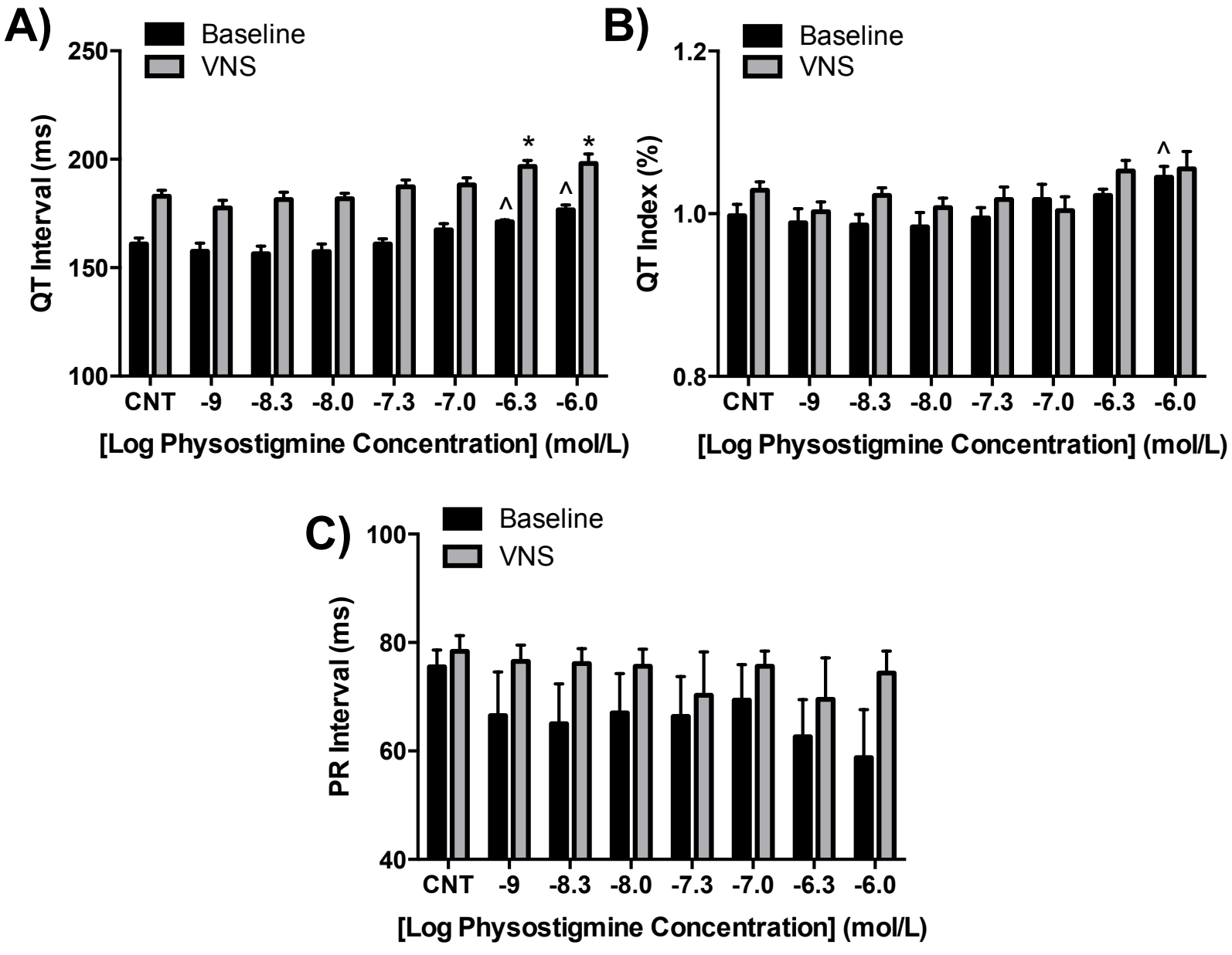

Figure 3

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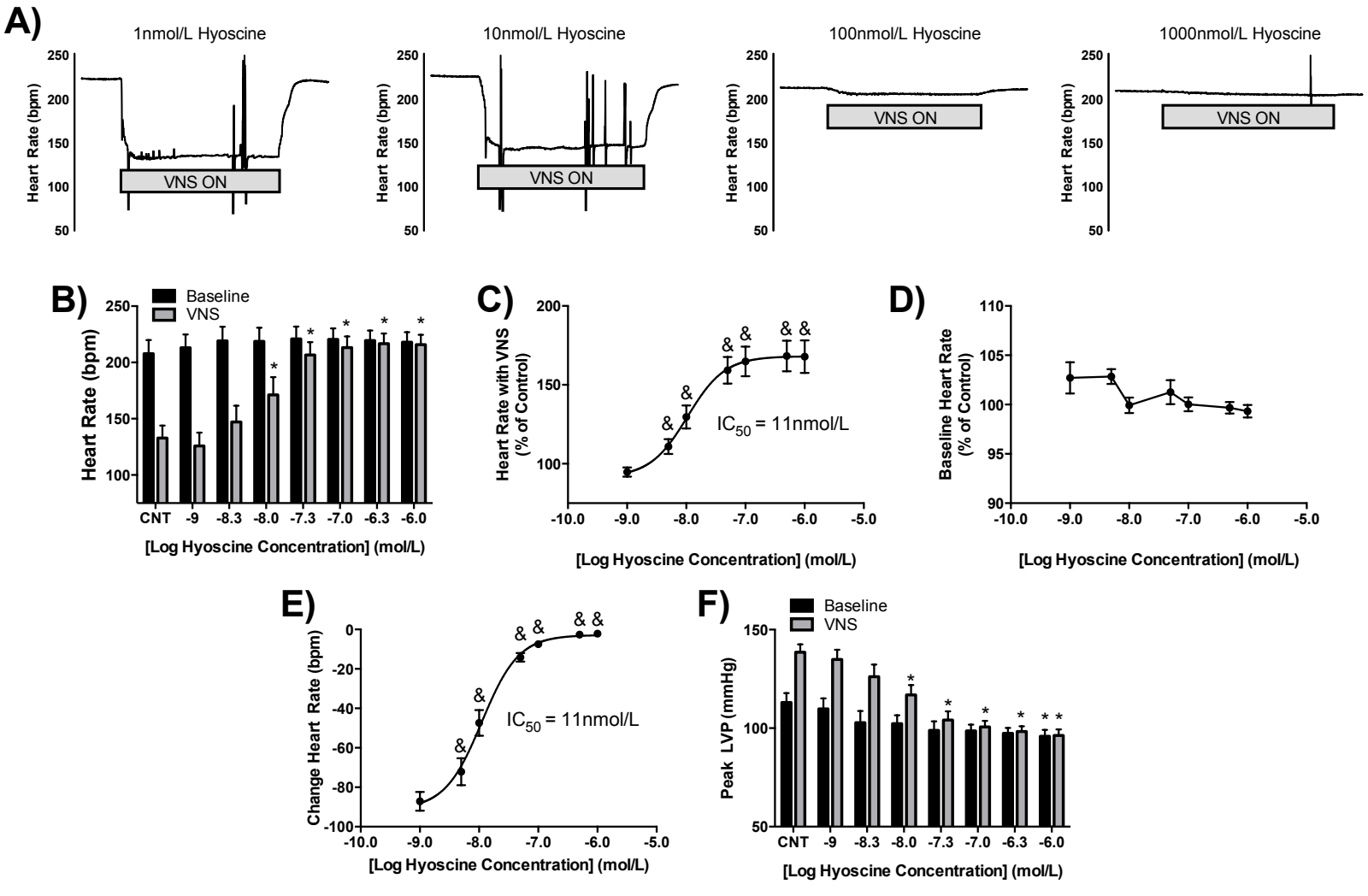

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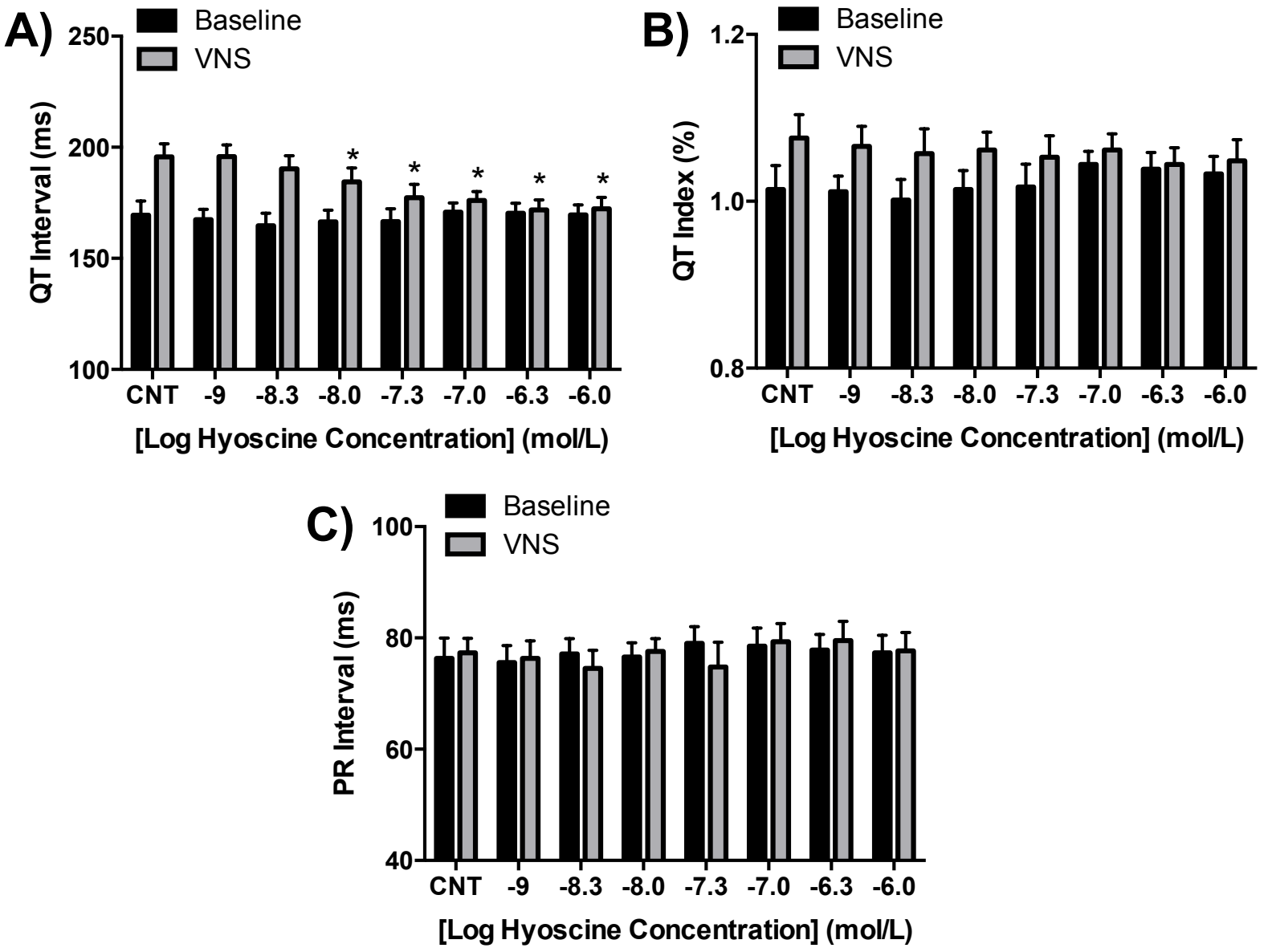

Figure 5

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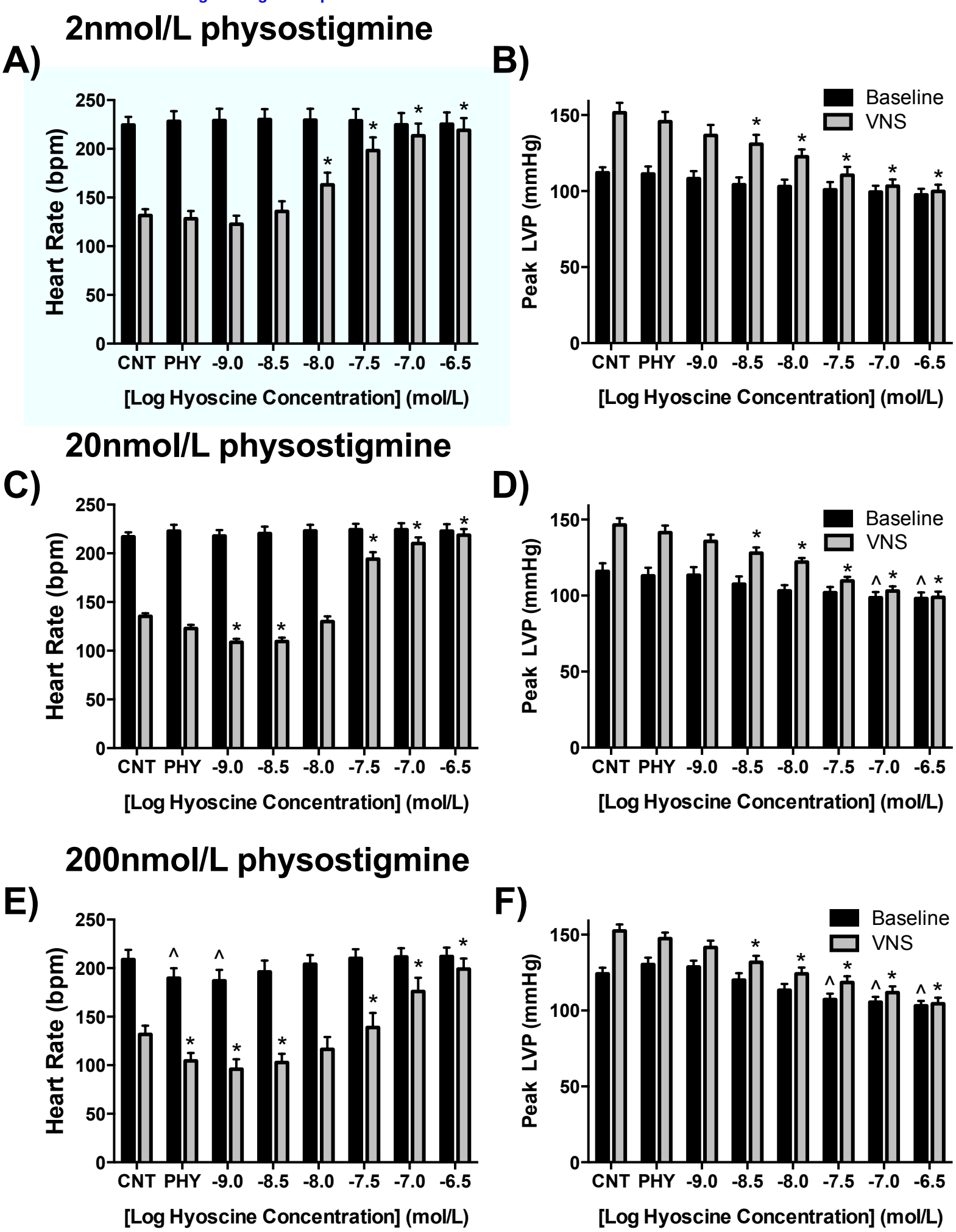

Figure 6

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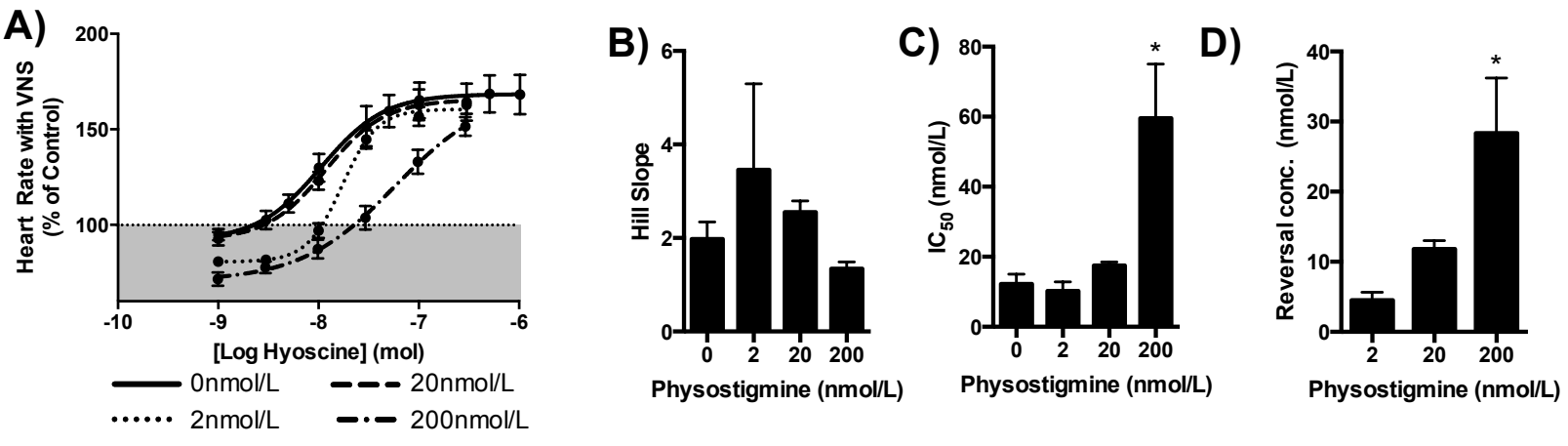

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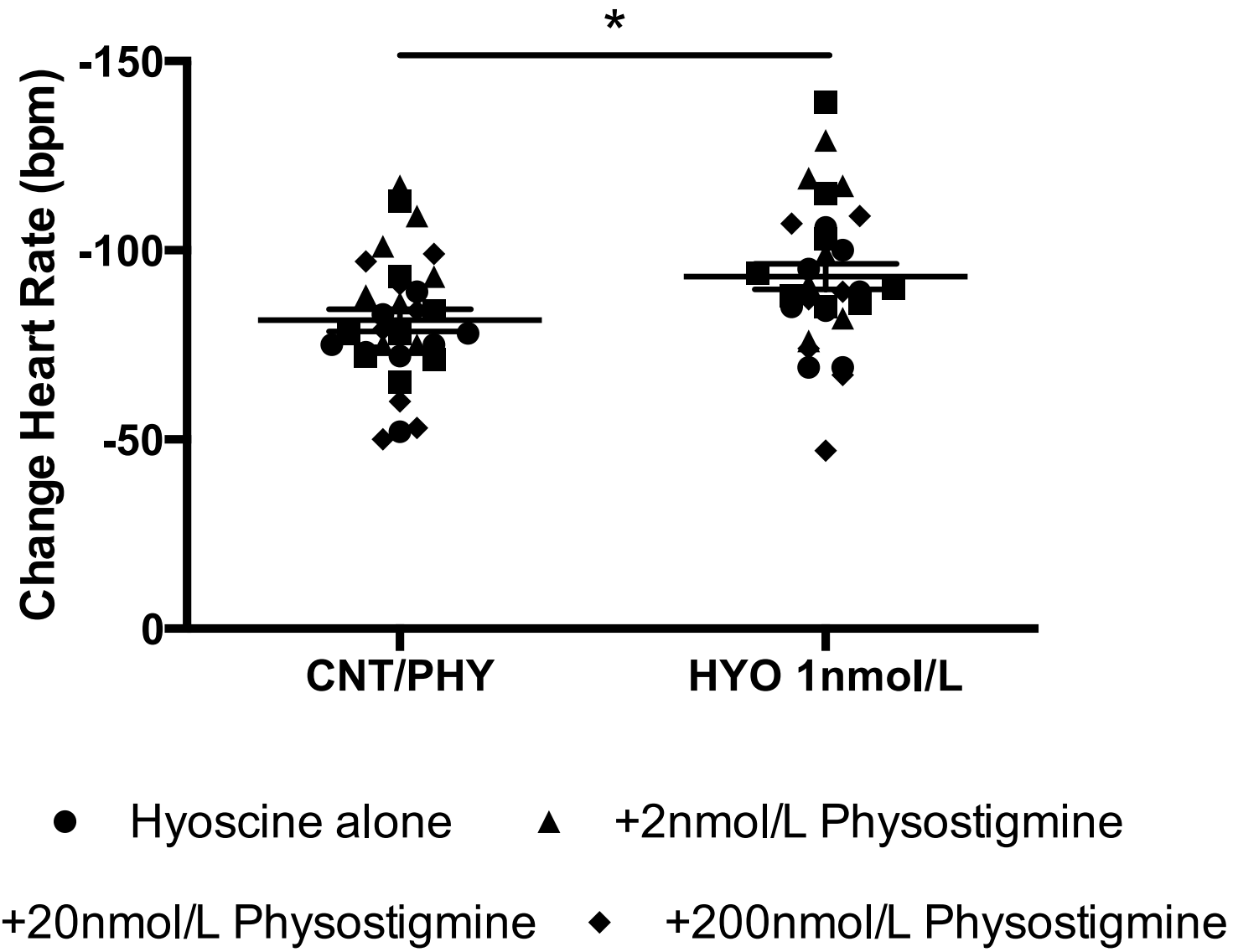

Supplementary Material

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